Neuroblastoma Screening in Infants Postponed After the Sixth Month of Age: A Trial to Reduce "Overdiagnosis" and to Detect Cases With "Unfavorable" Biologic Features

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Background. Encouraged by Japanese reports of the benefits of screening 6-month-old infants for neuroblastoma, a neuroblastoma screening program was introduced in Austria in 1991. However, because of concerns related to "overdiagnosis" by screening at this age, the screening test was performed at a later age.

Methods. From March 1991 to February 1995 neuroblastoma screening was performed on filter paper urine specimens in 100,043 Austrian infants (median age 8.5 months). Primary analysis of urine catecholamines (vanillylmandelic acid and homovanillic acid) was performed by use of an EIA method. Questionable or positive results were confirmed by high performance liquid chromatography (HPLC). A double retest was requested following a positive HPLC result.

Results. Twenty-one infants were admitted to a hospital following repeatedly elevated values of vanillylmandelic acid (VMA) and/or homovanillic acid (HVA). Eleven infants were found to have neuroblastoma (three stage 1, four stage 2B, four stage 3). Treatment consisted of surgery alone with total or subtotal resection in eight cases, surgery and chemotherapy in two cases, and chemotherapy alone in one case. Biologic features were assessed in all tumors excluding ploidy in one case. The majority of the tumors analyzed were near-triploid (9/10), however, two tumors revealed N-*myc* amplification.

Conclusion. Our results demonstrate that stage distribution and biologic features of neuroblastomas diagnosed by screening at 8.5 months are different from the results of screening at 6 months. Furthermore, the detection of one neuroblastoma among 9,100 screened infants is significantly lower than the incidence of the Japanese screening program. Our results suggest that screening at an age of 7 to 10 months reduces overdiagnosis and may be of more benefit than earlier screening. Med. Pediatr. Oncol. 29:1–10, 1997.

Key words: neuroblastoma screening; urine catecholamines; VMA; HVA; EIA; high performance liquid chromatography (HPLC); biologic features; N-*myc;* genetics; ploidy

INTRODUCTION

Screening for neuroblastoma started in Japan more than 20 years ago [1,2]. It has been demonstrated that many neuroblastoma cases can be detected by urine mass screening [1–7]. Encouraging reports [2,5] describing the beneficial effect of neuroblastoma screening have led to pilot studies in several countries or regions [8–10]. Some investigators proposed that neuroblastoma screening should be performed as soon in life as possible [11,12]. The North America/Quebec project introduced additional screening at an age of 3 weeks [12,13].

In 1990 it was decided to start a neuroblastoma screening program in Austria, too [8]. At that time, however, the first critical reports about neuroblastoma screening were published [14–20]. These reports emphasized the ''overdiagnosis'' of neuroblastoma cases by early screening, and some authors argued that neuroblastoma screening at an age of 6 months might predominantly detect neuroblastomas that would never be diagnosed clinically, i.e., tumors that would regress spontaneously [18,20–22]. In fact, the introduction of early neuroblastoma screening has led to an overdiagnosis of this disease and neuroblastoma incidence has doubled in screening areas [22–27]. Analysis of the biologic features of neuroblastomas detected by the Japanese screening

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Received 1 February 1996; Accepted 6 May 1996

programs and those presenting clinically showed remarkably differing findings [15,19,28–33]. In addition, children developing neuroblastoma after the 6-months test were frequently missed by early screening [14,15,18,19]. The reports concluded that screening at an age of 6 months could not be generally recommended [17,18,20]. Some authors argue that neuroblastoma mortality may be little affected by early screening [15,34]. Thus, the need for prospective controlled studies was emphasized [35], but these studies require huge numbers of screened and unscreened infants [36].

It was recently suggested that neuroblastoma screening should be postponed to a later age [14,27] to concentrate on unfavorable tumors. However, the optimal age for neuroblastoma screening is not yet known and it remains questionable whether such an optimal age really exists.

An optimal screening program should (1) detect only cases which would not regress spontaneously and therefore should not lead to an increased incidence, (2) not miss cases which present clinically at a later age, (3) lead to an ''early'' diagnosis of the ''bad subset,'' and (4) improve the prognosis of this disease. It is questionable whether a single screening test at any age will be able to fulfill all these criteria. Repeated screening (e.g., at 6 and 12 months) would miss fewer cases, but again would lead to over-diagnosis and incur additional costs. A single late screening (e.g., at 18 months) most likely would avoid over-diagnosis, but would be too late for many unfavorable neuroblastoma cases. Thus, the optimal age for a single screening test may be between the 6th and 18th month of life.

Therefore, screening age was postponed to the 7th to 10th month in the Austrian project. It was the aim of this study to investigate whether screening after the age of 6 months could reduce overdiagnosis and would enable detection of neuroblastoma cases with ''unfavorable'' biologic features.

PATIENTS AND METHODS

Collection of Samples

In February 1991, all pediatricians and general practitioners of Austria (7.8 million inhabitants, 84,000 km^2, 94,000 newborn infants per year) were invited to participate in the neuroblastoma screening program. Additionally, local health boards were asked to support the screening program by information campaigns and by distributing filter paper strips to pediatricians and general practitioners for urine collection. Screening was on a totally voluntary basis and therefore strongly dependent on the doctors' collaboration.

Filter papers (Schleicher & Schuell, Munich, Germany) were distributed by pediatricians and general practitioners to parents of infants aged 7 to 9 months on the occasion of a routine baby check-up. Parents were asked through a written leaflet to insert the filter paper into the napkin for urine collection, to dry it well afterwards, and to send it directly to the laboratory.

Analysis of Urine Catecholamines

Urine was eluted from filter paper by phosphate buffer base solution (PBBS). The samples were analyzed for vanillylmandelic acid (VMA), homovanillic acid (HVA), and creatinine. For quantitative analysis of VMA and HVA, a new EIA method (Yamasa Shoyu Co., Choshi, Japan [37,38]) was used as the primary method and creatinine was determined by the Jaffe reaction. In case of too diluted samples (creatinine < 10 mg%) a retest was requested from parents, highly concentrated samples (creatinine > 100 mg%) were further diluted. The analysis was fully automated by a Beckmann (Fullerton, CA) work station combined with a side loader. For photometric assessment an Eurogenetics plate reader connected to a data station was used. Data acquisition, calculations, and data storage were performed by a self-developed DBASE software package. Cut-off values had been determined by the mean value plus 2.5 times the standard deviation and were set at 26 μg/mg creatinine for VMA and 30 μg/mg creatinine for HVA. False-positive results, especially for HVA, were most likely caused by cross reactivity, and had been excluded from calculations of cut-off values.

When a positive EIA result was obtained, high performance liquid chromatography (HPLC) using Bio-rad equipment (Richmond, CA) was performed. Again cut-off values had been predetermined and were 20 μg/mg creatinine for VMA and 37 μg/mg creatinine for HVA.

Retests and Hospital Admission

In cases in which elevated values for both EIA and HPLC methods were obtained, a double retest was requested. Parents were contacted by a letter and asked to collect two further urine samples on the same day, one in the morning and one in the evening. Parents were advised to avoid banana and vanilla in the baby's food prior to urine collection. Retests were analyzed by both EIA and HPLC methods.

In cases with two positive results, parents were informed by phone and a letter about the results of the tests. Parents were asked to attend the local children's hospital. An enclosed letter was addressed to the pediatrician of the hospital concerned, asking for further investigations including abdominal ultrasonography, chest X-ray, serum analysis, and a 24-hour urine collection.

Further Treatment

If a tumor was found by sonography and/or X-ray, further diagnostic work-up and treatment was initiated by the local oncologist, in most cases according to the Aus-

trian treatment protocol for neuroblastoma patients (A-NB87 [39] and A-NB94, respectively). Some cases with incomplete tumor resection received no chemotherapy. This practice was applied only in cases with favorable biologic markers and in expectation of spontaneous regression of the residual tumor [40–42].

In cases with negative findings by sonogram and X-ray, further investigations were dependent on laboratory findings. If further urine catecholamines were not elevated, no further investigations were performed. In cases with ongoing elevated urine catecholamines and/or elevated serum NSE levels, further investigations were performed (computerized tomography and/or MIBG scintigraphy and/or bone marrow puncture). If these investigations revealed no tumor, further examinations of urine catecholamines were continued until the levels returned to normal.

Staging and Analysis of Tumor Material

Detected neuroblastomas were staged according to the Evans and INSS classification [43,44]. Primary resection of the tumor was attempted. When the tumor was not resectable a biopsy was recommended. Histologic classification according to Shimada et al. [45] was performed in nine cases.

Biologic features were determined in all tumors. Tumor samples were resuspended in RPMI 1640 plus antibiotics and 10% fetal calf serum. R-banding was performed by employing a chromomycin/distamycin/DAPI staining technique. Double fluorescence in situ hybridization (FISH) analyses were carried out on cytospin slides and on touch preparations prepared from resected tumors or biopsies. To evaluate the integrity of the short arm of chromosome 1, the number of centromere signals pUC1-77 (D1Z1) and telomere signals of chromosome 1 demonstrated with the VNTR probe p1-79 (D1Z2) specific for the subtelomeric region of 1p (1p36.33) was evaluated in at least 100 nuclei. Hybridization conditions and detection of the hybridized probes were performed as reported previously [46,47]. The same procedure was performed to evaluate the copy number of the N-*myc* oncogene. The labeled DNA probe pNb9 (kindly provided by Dr. Schwab) specific for the N-*myc* gene was used in combination with the centromere 2 specific probe D2Z (Oncor, Gaithersburg, MD). Results of the FISH analysis for N-*myc* copy number were confirmed by Southern blotting using pNb1 as specific probe (kindly provided by Dr. Schwab). Ploidy of tumor cells was determined by flow cytometry according to standard conditions using a FACStar flow cytometer (Becton Dickinson, San Jose, CA).

Tumor registry, Neuroblastoma Incidence, and Follow-Up

Since 1987 Austrian neuroblastoma patients are treated according to the national study protocols A-NB87 and A-NB94, respectively [39] (Ladenstein and Mutz, unpublished results). All neuroblastoma patients are registered by the study center and an ''active'' follow-up is performed. The hospital where the child is (was) under treatment is contacted periodically and information about the actual status is requested.

Neuroblastoma incidence in Austria was compared for two 4-year periods (1987–1990 and March 1991–February 1995, respectively). For calculation of the incidence, neuroblastoma cases observed during each period were related to the number of liveborn infants of the same period. Neuroblastoma cases were divided into two groups with respect to the age at diagnosis (<12 months and >12 months, respectively). Furthermore, neuroblastoma incidence of the screening group was compared with the incidence of the unscreened group.

In order to find possible false-negative screening results (patients who had a normal screening test result, but developed neuroblastoma later), data of the Austrian neuroblastoma registry were compared with screening data.

RESULTS

Compliance

Between March 1991 and February 1995 100,043 samples of 7- to ten-month-old infants were received by our laboratory. There were 375,612 liveborn infants in Austria over this period. Hence, compliance with the screening program was 26.6%. Compliance rates varied between regions with a range from 12 to 75%. These differences were mainly a consequence of the different appreciation of the screening program by the local authorities and health boards.

Results of EIA and HPLC Analyses

As shown in Figure 1 the primary EIA analysis revealed a clear negative result in 89,139 (89.1%) samples; 4,208 (4.2%) samples were inadequate, most frequently due to too little urine, insufficiently dried samples, and stool contamination. The remaining 6,696 (6.7%) specimens gave a positive EIA result and were reanalyzed by HPLC. Nine hundred four (0.9%) samples showed a positive result by both EIA and HPLC methods, and required a retest. The double retest (one sample collected in the morning, another in the evening) confirmed elevated urine catecholamines in 21 infants (0.02% of all investigated infants). These infants were admitted to the local children's hospital.

Clinical Investigation

Twenty-one children underwent clinical investigation following a double positive screening test result. In ten cases no neuroblastoma was found by clinical investigation. Six of these infants had normal urine catecholamines under the conditions of clinical urine collection.

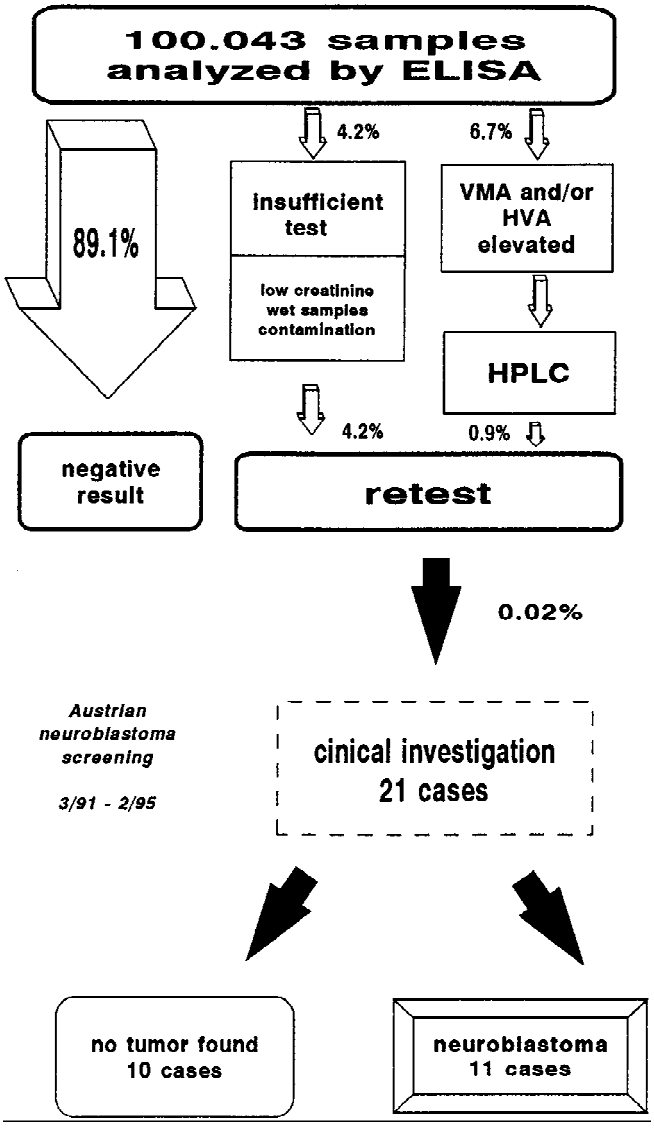

Fig. 1. Flow sheet of the 100,043 urine samples analyzed by the Austrian neuroblastoma screening program between March 1991 and February 1995.

Abdominal ultrasonography, chest X-ray, standard laboratory examinations (including LDH, NSE, and ferritin), but no further investigations were performed in these cases who were then discharged. In four cases urine catecholamines were found to be elevated and in three cases were associated with elevated serum NSE (>15 μg/l). Again, no neuroblastoma was found even after intensified investigations (MIBG scan, bone marrow aspirates, and computerized tomography of chest and abdomen). Follow-up analyses of further urine samples were performed for these infants and showed normalization of values 4, 5, 10, and 13 months after the first sample collection.

In the 11 remaining cases (0.01% of all screened infants), neuroblastoma was confirmed by clinical investigations. The median delay from the time of first screen to the diagnosis was 4 weeks (2–8 weeks). Interestingly, three of these tumors were not seen by the first ultrasonography despite the stated suspicion of neuroblastoma by screening. Two of these tumors were detected by a second ultrasound examination while one tumor was found by computerized tomography only.

Characteristics of Neuroblastoma Cases Detected by Screening

Stages. According to the Evans classification, two cases were classified as stage I, two were stage II, seven were stage III. In contrast, the INSS classification defined three tumors as stage 1, four tumors as stage 2B, and four as stage 3.

Treatment. In 9/11 cases, a primary surgical treatment was performed. Unfortunately, one of these infants died after operation due to a surgical incidence (aortic rupture). In four cases, the tumors were totally (macroscopically and microscopically) resected; nevertheless, postoperative chemotherapy was administered in one of these cases. In one case, microscopic residual tumor was left, but the patient remained without further therapy. In three cases, small macroscopical residual tumors remained, but no chemotherapy was administered according to the more recent protocol guidelines [40].

In the remaining two cases total or subtotal resection was impossible due to tumor size and site. These two infants underwent chemotherapy after initial biopsy, and one of them had a second-look operation.

Histologic Classification, Laboratory Findings, and Biologic Features

One tumor was not classified due to insufficient collection of tumor material by needle biopsy. The histologic classification according to Shimada revealed 8/9 ''favorable'' and 1/9 ''unfavorable'' cases.

Nine out of ten tumors were near-triploid, but cytogenetic analyses showed structural chromosomal aberrations in two cases besides gains of whole chromosomes. The DNA index of one tumor revealed three peaks in the near-tetraploid chromosome range.

FISH analysis for detection of 1p36 deletion was performed in all tumors (11/11) and none showed loss of genetic material for the band 1p36.33 of the short arm of chromosome 1.

N-*myc* analysis by Southern blotting and FISH analysis revealed a 5- to 30-fold and 20- to 100-fold amplification, respectively, in two tumors. In nine tumors no change in copy number of this proto-oncogene could be determined.

Neuron specific enolase (NSE) was elevated (>15 μg/l) in 10/11 patients. Serum lactate dehydrogenase (LDH) was elevated (>360 U/l) in 4/11 cases and borderline in

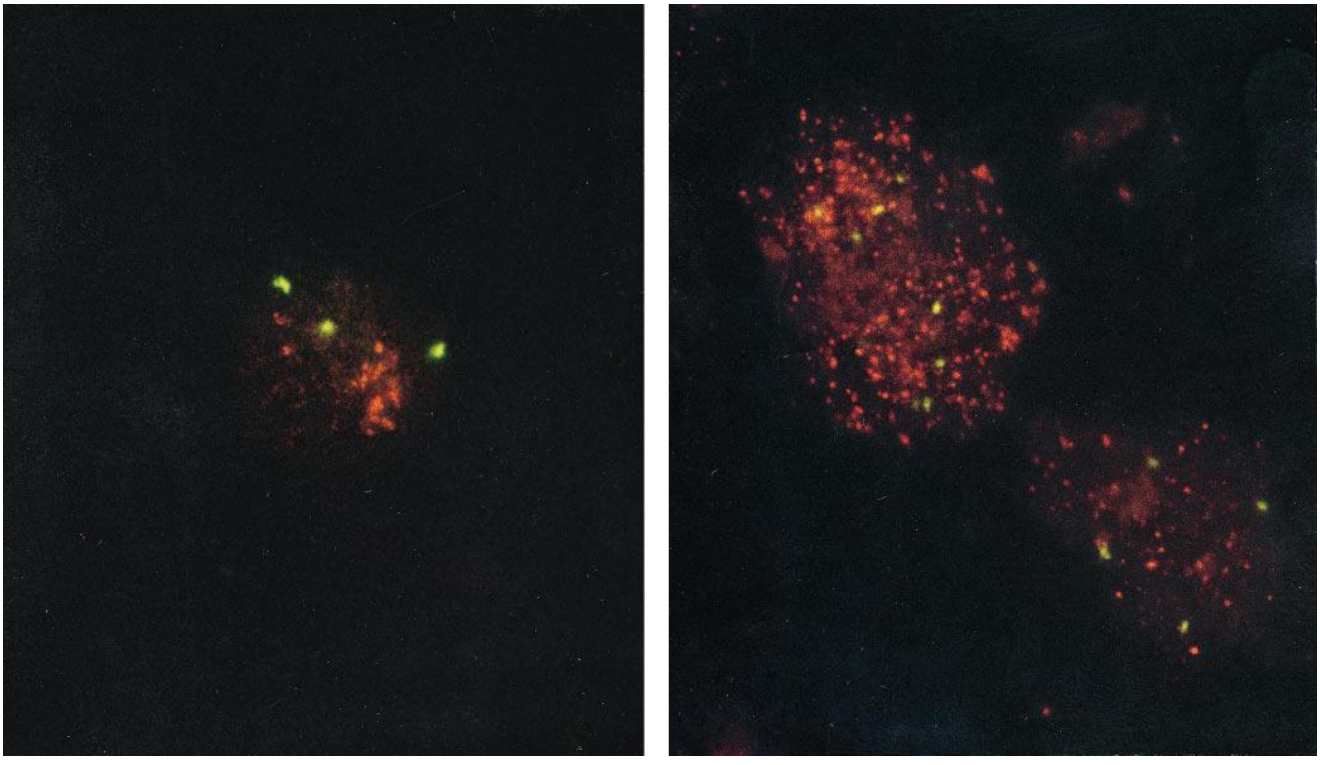

Fig. 2. In situ hybridization with a N-*myc* probe to evaluate the copy number of this oncogene. **a:** A nucleus from the tumor of patient 1 displaying two single red hybridization signals and a cluster of approximately 10 hybridization spots (red) clearly indicate an amplification of the N-*myc* oncogene organized in an homogeneously staining region (HSR). The tumor cells of patient 8 presented with 20 to 100 hundred copies of the N-*myc* oncogene per nucleus. A typical example is shown **(b)** where approximately 50 hybridization spots can be identified in both central nuclei; the third nucleus displays approximately 20 N-*myc* hybridization spots. The green signals highlight the centromeres of chromosome 2 and thus represent the number of chromosomes 2 per nucleus (on average three signals per nucleus).

another case. Serum ferritin was in the normal range in all cases.

Follow-Up and Outcome of Screened Patients

Follow-up time and outcome for the patients diagnosed by the Austrian neuroblastoma screening program are listed in Table I. As of November 1995, the median follow-up time is 20.5 months. Apart from the one patient who died after a surgical complication, all patients are alive and without major problems. One patient (patient 6) developed severe septicemia due to chemotherapy induced neutropenia, but has totally recovered.

The small residual tumors in patients 3 and 9 showed total regression while in patient 10 the size of residual tumor is decreasing. The microscopic residual tumor in patient 11 remained undetectable by postoperative magnetic resonance images and MIBG scan. In patient 4 only a small ''silent'' tumor remains after chemotherapy (VGPR). All patients with complete tumor resection are well and free of disease.

Search for ''False-Negative'' Cases

The data of the Austrian neuroblastoma registry were compared with the data of the 100,043 screened infants by name and date of birth. No ''false-negative'' case has been found so far. Median follow-up time for screened infants as of November 1995 is 26 months. Thus, as the median age at screening was 8.5 months, the median age of the screened children is 34.5 months.

Neuroblastoma Incidence Before and After Onset of Screening

1987–1990 Neuroblastoma incidence in Austria was 1/6,000 (59 neuroblastoma cases, 353,768 liveborn infants). At diagnosis 41/59 patients were older than 12 months (10.2 cases per year).

TABLE I. Neuroblastoma Cases Diagnosed by the Austrian Screening Program (1991–1995)*

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age (months)	9,1	8,9	10	11,8	8,8	8,9
Stage	III (3)	III (1)	III (3)	III (3)	II (2B)	III (3)
Localization	retroperiton.	Adrenal	retroperiton.	retroperiton.	retroperiton.	Adrenal
Tumor volume (ml)	280	175	45	175	32	18
Stage explanation	midl. +, unres.	midl. +, tot.res.	L.n. +, resid.tu.	midl. +, unres.	L.n. +, tot.res.	midl. +, unres.
Symptoms	—	—	—	—	—	—
VMA/HVA (ELISA)	301/198	66/55	40/41	168/86	35/39	50/31
VMA/HVA (HPLC)	n.d.	55/50	29/53	146/106	35/72	38/29
VMA/HVA ratio (HPLC)	1,5	1,1	0,5	1,4	0,5	1,3
NSE	136	59	28	37	14	20
Ferritin	82	17	24	6	18	35
LDH	512	482	361	277	278	252
Ploidy-cytogen.	3n, str.aberr.	4n	3n	n.d.	3n	3n
N-myc ampl.	5x–30x	No	No	n.d.	No	No
1p deletion	No	No	No	No	No	No
Treatment	su.	su., chemo	su.	Biopsy, chemo	su.	Biopsy, chemo, su.
Shimada	fav.	fav.	fav.	n.d.	fav.	n.d.
Outcome	dead, surg.com.	NED	NED	VGPR	NED	NED
Follow-up (months)	0	33	27	25	23	21

March 1991–February 1995 Neuroblastoma incidence increased to 1/5,220 (72 neuroblastoma cases, 375,612 liveborn infants). At diagnosis 30/72 patients were older than 12 months (7.5 cases per year).

Incidence in Screened and Unscreened Children

Between March 1991 and February 1995 in Austria 72 neuroblastoma cases were diagnosed in children under 15 years. Twenty-eight of these cases (39%) were diagnosed before infants reached the median screening age (8.5 months); one of these patients was diagnosed by screening at the age of 8.3 months. Three of the 28 infants died before the age of 8.5 months and two further infants died before the age of 9.5 months (= median age at diagnosis in screened infants).

Forty-four of the 72 neuroblastoma cases (61%) were diagnosed in children over 8.5 months. Twenty-eight of these 44 patients were born after July 1990 and therefore had the chance to be screened. Ten of these 28 cases were diagnosed by screening among 100,043 screened infants (1/10,000) whereas the remaining 18 cases were diagnosed among 275,483 unscreened children born between July 1990 and June 1994 (1/15,300).

DISCUSSION

The question ''Do children benefit from neuroblastoma screening'' is of major importance [20]. Whereas some Japanese reports describe a decrease in the incidence of cases of advanced neuroblastoma in older children after the introduction of neuroblastoma screening [4–6], other authors doubt about the efficiency of screening at 6 months [17–20,22,34]. The question whether screening at 6 months of age can reduce mortality from neuroblastoma has not yet been answered with statistical significance and requires a huge controlled study [35,36]. However, a significant increase of neuroblastoma incidence in areas with early screening (6 months or earlier) probably means that many infants are treated unnecessarily. For these infants screening performance is of disadvantage and some infants—as one of our patients—even may die due to perioperative problems or side effects of chemotherapy. These considerations were taken into account by the Austrian screening program in 1991 inasmuch as screening was postponed to an infant's age of 7 to 10 months [8].

Although the Austrian screening program was not supported by official boards and therefore has to be considered as a ''pilot project'' to test feasibility, the results of this program are different from those obtained by other screening programs [19,29,32,33]. As far as our own screening program is concerned, the incidence of detected neuroblastomas was lower than the incidence of the Japanese program [22,27] and comparable to the North America/Quebec project [25]. The introduction of screening in Austria was parallelled by a slight increase of neuroblastoma incidence. However, in contrast to Japanese programs [18,22] a corresponding decrease in the rate for children at older ages (>12 months) was observed. The observation that almost 40% of cases were diagnosed before they reached the median screening age suggests that increased incidence is rather a consequence of incidentally diagnosed cases in early infancy [48] than a consequence of additionally diagnosed screening cases. The observation of a higher neuroblastoma incidence in screened children over 8.5 months (1/10,000) than in unscreened children of the same age group (1/15,200) does not necessarily mean that this is a consequence of ''overdiagnosis,'' because it is the aim of the screening program to detect cases earlier. The higher incidence in

TABLE I. (Continued)

	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11
Age (months)	9,5	8,3	10,4	10,3	9,5
Stage	III (2B)	I (1)	I(1)	III (2B)	II (2B)
Localization	retroperiton.	Adrenal	retroperiton.	retroperiton.	Adrenal
Tumor volume (ml)	20	48	14	70	115
Stage explanation	L.n. +; midl. +	tot.res.	resid.tu.	midl. +, resid.tu.	m.resid.tu., L.n. +
Symptoms	—	—	—	—	—
VMA/HVA (ELISA)	53/49	67/93	35/24	67/33	88/68
VMA/HVA (HPLC)	37/45	53/72	32/28	59/50	170/139
VMA/HVA ratio (HPLC)	0.8	0.7	1.1	1,2	1,2
NSE	45	75	40	34	34
Ferritin	66	121	47	12	29
LDH	379	465	292	294	334
Ploidy-cytogen.	3n	3n	3n	3n	3n, str.aberr.
N-myc ampl.	No	20x–100x	No	No	No
1p deletion	No	No	No	No	No
Treatment	su.	su.	su.	su.	su.
Shimada	fav.	unfav.	fav.	fav.	fav.
Outcome	NED	NED	NED	regress.	NED
Follow-up (months)	20	17	13	12	12

*Characteristics of the 11 neuroblastoma cases detected by the Austrian screening program between March 1991 and February 1995. midl.+ = tumor infiltrating across the midline; unres. = unresectable; tot.res. = total resection; resid.tu. = macroscopic residual tumor; m.resid.tu. = microscopic residual tumor; L.n.+ = positive lymph nodes; 3n = tripoidy; 4n = tetraploidy; str. aberr. = structural aberrations; fav. = favorable; unfav. = unfavorable; surg. = surgery; surg. com. = surgical complications; chemo = chemotherapy; NED = no evidence of disease; VGPR = very good partial response.

the screened group could be a temporary phenomenon due to a short observation period, and a longer follow-up time is needed.

It is noticeable that no false-negative cases have been observed so far in the Austrian project. As the median age of children who previously underwent screening is now 34.5 months, it can be supposed that tumors found by our screening program were at least in part those which would have presented clinically at a later age.

Concerning stage distribution there were relatively more cases with advanced localized stages than in the Japanese results [19]. According to the Evans classification 7/11 tumors were classified as stage III and according to INSS criteria 4/11 tumors were stage 3. In contrast to the Japanese and the North America/Quebec screening programs, no stage 4 and no stage 4s disease was diagnosed in Austria by screening. The lack of stage 4 may be related to the relatively small number tested.

It was one aim of the Austrian study to detect cases with ''unfavorable'' biologic features. Recently, there is a controversial discussion concerning the predictive relevance of different biologic features. Whereas N-*myc* gene amplification has been frequently described as an independent ''unfavorable'' prognostic marker [49,50], a recent study reports lack of correlation N-*myc* gene amplification with prognosis in localized neuroblastoma [51]. A similar controversy exists for the 1p deletion and di-/tetraploidy. Some authors describe these features as independent prognostic factors [52,53] whereas an independent role could not be found by other investigators [49,50]. These results indicate that adequate therapy can overcome the genetically unfavorable markers at least in some instances. This notion is supported by the good response to treatment by chemotherapy in approximately 30% of the cases despite unfavorable genetic markers. Genetic markers are nevertheless powerful indicators of aggressive tumor biology [46].

Neuroblastoma cases found by the Japanese screening program almost uniformly presented with favorable biologic markers, and worldwide only a few ''unfavorable'' cases have been detected by screening [33,54,55]. In the Austrian pilot project, more cases with locally advanced disease were found, and 4/11 cases had at least one feature that may be unfavorable. Two of the 11 infants diagnosed by screening had N-*myc* amplification, two tumors showed cytogenetic aberrations, and one tumor was near-tetraploid.

DNA content of both N-*myc* amplified tumors was in the triploid range. This number of tumors with N-*myc* amplification despite a near-triploid DNA content is in agreement with the figure of approximately 18% of triploid tumors with N-*myc* amplification and/or 1p deletion found in neuroblastomas [46] (Ambros, unpublished results). Unfortunately, one of our two patients with N-*myc* amplification died as a consequence of a surgical complication. In the other patient, despite the presence of high N-*myc* amplification, therapy consisted only of complete surgical resection. At the time of this report the patient has been well for 17 months. Although it cannot be proved, it suggests that this patient did benefit from screening as the tumor was detected before metastatic spread happened.

A recent report on children with N-*myc* gene amplified tumors [51] shows two patients surviving after total tumor resection (stage A). Further two patients with stage B tumors survived after surgery and chemotherapy. However, two children died (one stage A, one stage B) despite surgery and chemotherapy. These results are in line with our findings [46] and confirm a general oncologic experience that the chance to cure an individual patient is not only dependent on the tumor's biology and aggressiveness, but also on tumor stage (resectability) and therapy.

The majority (9/10) of our screening cases studied for ploidy were in the near-triploid chromosome range. The high proportion of triploid tumors is in line with the figures given by Hayashi et al. [29] and Kaneko et al. [15] who found in a screened population 100 and 80%, respectively, of the tumors in the triploid chromosome range. However, Kusafuka et al. [56] reported nearly 25% diploid/tetraploid tumors in infants aged 6 months. As the authors found a high discrepancy between favorable outcome and diploid/tetraploid DNA content, they concluded that a di-/tetraploid DNA content is not predictive of poor outcome in individual patients. Unfortunately, only limited data on therapy and surgical completeness are given. The clinical impact of di-/tetraploidy on the patients' outcome certainly needs further detailed studies, ideally in studies where no chemo- or radiation therapy is given even in residual tumors, e.g., in the Localised Neuroblastoma European Study (LNESG [40]). In addition, two different methods to evaluate the DNA content should be used to rule out any methodical difficulties and misinterpretations of the results obtained due to a high content of normal cells (e.g., lymphatic tissue, Schwann cells) in the tumor sample under investigation [57]. However, the finding of near-triploidy without the knowledge of other pertinent biologic markers is certainly not sufficient evidence to justify a reduction or omission of any cytotoxic therapy, especially as we experienced tumor progression in five unscreened patients with localized neuroblastomas (two of them had triploid tumors with N-*myc* amplification and/or 1p36 deletion [46]).

Another powerful prognostic indicator besides the N-*myc* copy number is the deletion at the short arm of chromosome 1. None of our screened patients presented with this aberration. The 1p deletion is the most frequent structural alteration in stage 4 tumors [53] and also in localized or stage 4s tumors which progressed later on into stage 4 tumors [46]. Interestingly, this aberration has never been observed in tumors undergoing spontaneous regression or maturation [46,57–59]. However, the predictive relevance of the 1p deletion as an independent prognostic marker has still to be clarified [49,50,53].

In three of our patients with primary surgical treatment, macroscopical residuals were left. However, all biologic features (N-*myc,* integrity of 1p36, ploidy) were favorable in these cases, and no chemotherapy was administered. In all three cases, the residuals showed spontaneous regression. This phenomenon was already described in previous and ongoing studies [40–42].

The Austrian screening program is unable to answer epidemiological questions concerning neuroblastoma mortality. Due to the population size, even with a 100% compliance rate a controlled study (50% screened, 50% unscreened) would take about 30 years to answer epidemiological questions concerning mortality with statistical significance [36]. Therefore, at the moment the Austrian study is continued as a ''pilot study'' with the aim to detect further neuroblastoma cases with unfavorable biologic features at an early clinical stage. Thus, the Austrian program may contribute to more knowledge about the ''natural course'' of neuroblastoma and to a better understanding of distinct entities of the disease.

CONCLUSIONS

Our results indicate that neuroblastoma screening after the 6th month of life is able to detect cases with ''unfavorable'' biologic features. The risk of overdiagnosis decreases after the 6th month of life. Furthermore, the rate of false-negative cases seems to be reduced and screening in late infancy might detect cases which would preferably progress and present clinically later in life. Screening in late infancy might, therefore, be of more benefit than early screening.

ACKNOWLEDGMENTS

This study was supported by the ''Kinderkrebshilfe,'' the Children's Cancer Funds of Styria and Carinthia, and the Styrian government. The authors express their thanks to Drs. J. Mann and L. Parker for critical reading the manuscript. The skillful help of Silvia Rumpler is greatly acknowledged. Our special thanks is dedicated to pediatricians and general practitioners who participated in this screening program by distribution of the screening test.

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